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Presence and diversity of Leishmania RNA virus in an old zoonotic cutaneous leishmaniasis focus, northeastern Iran: haplotype and phylogenetic based approach

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ABSTRACT

Objective: *Leishmania* RNA virus (LRV) is a double-stranded RNA (dsRNA) virus that circulates within many species of the *Leishmania* parasite. In this study, we aimed to investigate the presence of LRV2 circulating in *Leishmania* isolates in an old focus of ZCL located in northeastern of Iran.
Methods: *Leishmania* isolates were collected from 85 patients that confirmed to have cutaneous leishmaniasis (CL) based on parasitological examination. To identify the *Leishmania* isolates, species-specific primer sets were applied for molecular identification. The presence of LRV2 was performed by RdRp-semi nested-PCR. The genetic diversity were calculated using MEGA and DnaSP. To assess haplotype diversity, 31 LRV2 strains in different regions were surveyed using analysis a 292-bp section of the RdRp sequences.
Results: Out of 85 patients, 83 (97.6 %) were diagnosed with *L. major* and 2 (2.4 %) with *L. tropica*. LRV2 virus was detected in 59 (69.4%) of the CL cases. For the first time, LRV2 was reported in one *L. tropica* strain in Iran. The current LRV2 sequences indicated the highest similarities to an Old World LRV2. Moreover, 10 unique haplotypes were identified based on the analyzed sequences of the RdRp gene.
Conclusions: Our results indicated the highest occurrence of *Leishmania*/LRV2 co-circulation in this known ZCL focus from northeastern Iran. Phylogenetic analyses of LRV2 sequences confirmed that these isolates belong to the order of LRV2 from the Old World. This study offered an insight into LRV2 haplotype that the informative issue can be used for genetic research of LRV2 in other regions.

Introduction

Leishmaniasis is a neglected vector-borne disease caused by an intracellular protozoan parasite belonging to the genus *Leishmania* spp. (Alvar et al., 2012). Clinical presentations include a broad range of manifestations, ulcerative lesions of cutaneous leishmaniasis (CL), Mucocutaneous Leishmaniasis (ML), and visceral leishmaniasis (VL), also known as kala-azar (Desjeux, 2004). CL is the dominant manifestation and widely distributed worldwide,

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affecting approximately 1.5 to 2 million new cases each year and 350 million are at risk of acquiring the disease (WHO, 2018; Steverding, 2017). According to the World Health Organization (WHO), leishmaniasis is found in parts of the tropical and subtropical regions of the Far and Middle East, Central, Western and Eastern Europe and Africa as Old World leishmaniasis (OWL) and in South and Central America as New World leishmaniasis (NWL) (WHO, 2018; Steverding, 2017). It is found in about 98 countries, but approximately 95% of CL cases have occurred in seven countries, including Afghanistan, Algeria, Brazil, Colombia, Iran, Iraq and the Syrian Arab Republic (Reithinger et al., 2007). In Iran, there are two forms of CL, zoonotic cutaneous leishmaniasis (ZCL) caused by *L. major* and anthroponotic cutaneous leishmaniasis (ACL) caused by *L. tropica*. ZCL is endemic mostly in the rural and arid areas of about 80 percent of Iranian provinces (Ghatee et al., 2020). Golestan Province is an endemic province of ZCL in Iran, that the main causative agent of ZCL is *L. major* which is transmitted mainly by the *Phlebotomus papatasi Scopoli* and also the main reservoir host is *Rhombomys opimus* (Rassi et al. 2008; Sharbatkhori et al. 2014). Previous studies have shown that the incidence rate of ZCL has increased in the Golestan Province. In the study conducted by Sofizadeh et al. the highest incidence rate of the ZCL in Golestan Province were reported in Moraveh_Tappeh (121.5/100000) and Gonbad Kavoos (99.3/100000) respectively (Sofizadeh et al., 2016).

Almost three decades ago, the first *Leishmania virus*, also known as *Leishmania* RNA virus (LRV), was introduced in *L. guyanensis* (Tarr et al., 1988). LRVs are classified in the Totiviridae family composed of about 5.3 kb double-stranded RNA (dsRNA) genome, RNA-dependent RNA polymerase (RdRp) and capsid protein (Stuart et al., 1992). This virus particles are icosahedral (T = pseudo 2), non-enveloped, and approximately 30-50 nm in diameter (Stuart et al., 1992). According to the complete nucleotide sequences, LRVs were divided into two major groups: LRV1 infecting New World *Leishmania* (NWL) isolates such as *L. (Viannia) braziliensis* and *L. guyanensis* and LRV2 were identified in isolates from Old World *Leishmania* (OWL) such as *L. major.* (Scheffter et al., 1995; Saiz et al., 1998). Based on their phylogeny analysis, the complete nucleotide sequence of the RNA-dependent RNA polymerase (RdRp) gene indicates high diversity between LRV1 and LRV2 (less than 40% homology) (Scheffter et al., 1995).

In recent years, most studies focus on the topic of *Leishmania* RNA virus 1, because it is believed that it can increase parasite replication, pathology, and metastatic of lesions (Adaui et al., 2015). It was demonstrated that dsRNA of LRV interacts with Toll-like receptor 3 (TLR3), and induces pro-inflammatory cytokines and chemokines such as (interferon-β, TNF- α, CXCL10, CCL5, and IL-6) and subverts innate immunity. It, in turn, leading to a hyperinflammatory response to *Leishmania* infection (Hartley et al., 2012). However, the mechanisms that contribute to parasite persistence and increased disease remain largely undiscovered.

Previous evidence about the presence of LRV2 in *Leishmania* spp. isolates collected from Iran persuaded our study. The purpose of this current study was to determine the presence of LRV2 and its genetic diversity in *Leishmania* isolates , which circulated in the Golestan Province, as a known old focus of ZCL in the northeast of Iran.

Subjects and Methods

Ethical statement

This study was approved by the ethical principles and the national norms and standards for conducting Medical Research in Iran for the molecular identification of *Leishmania* RNA virus 2 (LRV2) in *Leishmania* parasites isolated from CL patients (IR.MAZUMS.REC.1398.344). Informed written consent was obtained according to guidelines from the adult subjects and from the children's parents and their written explanations were recorded using a structured questionnaire.

Study area

This study was conducted in Golestan Province with a population of about 1.9 million people covers the area of 20438 km^2 located in the northeast of Iran. It has a border of 348 km with Turkmenistan and is one of the known old foci of ZCL. Recent studies have been shown that the incidence of ZCL cases has increased in the last decade in the Golestan Province. The *Leishmania* parasites were collected from the patients before treatment who were suspected of CL have been referred to the referral health centers laboratory in Golestan Province, between July and December 2019 (Figure 1).

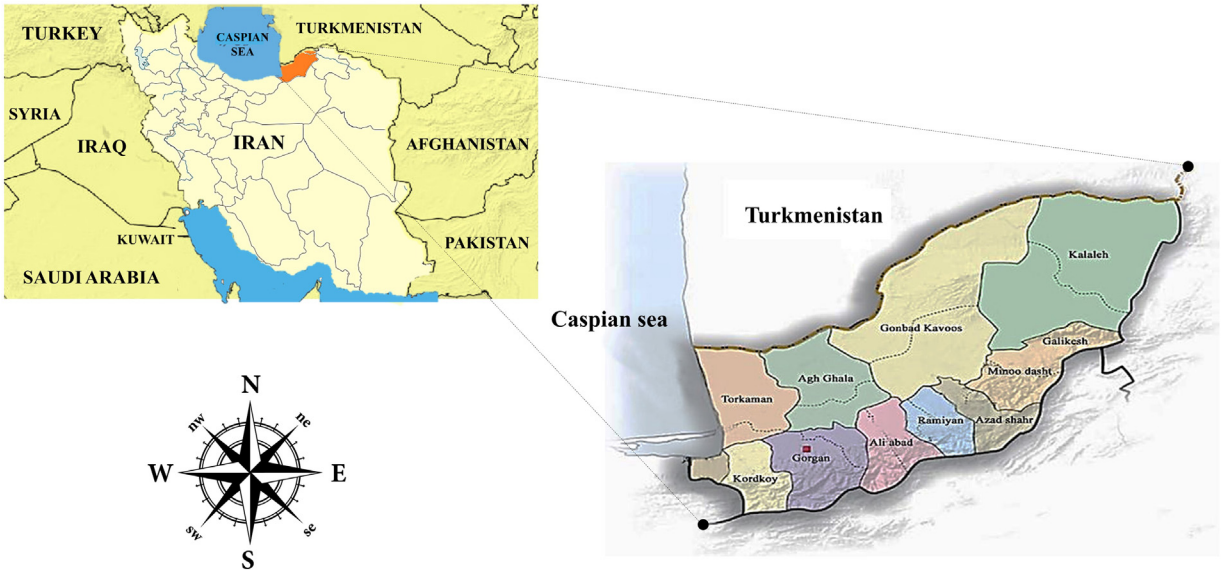

Figure 1. Representative images of map of the study area locations in Golestan Province as known old ZCL focus, in the northeast of Iran.

Samples collection

The dermal lesions were sterilized with 70% ethanol, and sample collection was done by scrapping the swollen edge of the lesions. The exudate materials were initially cultured on a Novy-MacNeal-Nicolle medium (NNN) medium. After 3-5 days, promastigotes were then transferred to RPMI 1640 medium (Gibco, Life Technologies GmbH, Germany) with 10% heat-inactivated fetal bovine serum (Gibco, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, German), and incubated at 26 °C.

Identification of Leishmania *species by PCR-RFLP*

The DNA of *Leishmania* was prepared using the Bioneer kit (Bioneer Company, Korea) according to the manufacturer's instructions. To amplify a fragment of approximate 1450–60 bp of the N-acetyl glucosamine-1-phosphate transferase (NAGT) gene in *Leishmania* genus parasites, specific primers Forward (5' TCA TGA CTC TTG GCC TGG TAG 3') and Reverse (5' CTC TAG CGC ACT TCA TCG TAG 3') were used (Saberi et al., 2018). The isolates studied were identified by PCRRFLP analysis and Acetylcoenzyme A carboxylase 1 (ACC1) (Xmil) enzyme (Fermentas GmbH, Thermo Scientific, Germany) that it can provide different digestion patterns for different species of *Leishmania* spp.

Viral double-strand RNA extraction

1×10^6 stationary phase promastigotes were collected by sedimentation, and for the extraction of viral dsRNA, 2 ml of lysis buffer [1 ml Tris-HCl (pH 8.0) and 10 % sodium dodecyl sulfate (SDS)] containing 100 μg/mL of proteinase K were added to the sediment of *Leishmania* promastigotes. The lysates were incubated at 37 °C, for 45 min. The lysates are then mixed with phenol, and centrifuged at 3000 rpm for 15 min. Carefully removal of the top aqueous solution, adds an equal volume of the ether, and centrifuged at 3000 rpm for 5 min. Removal as much of the top ether solution as possible and precipitated with 0.15 M NaCl in 70% ethanol, then washed and re-suspended in TNE buffer (100 mM NaCl, 1 mM EDTA (pH = 8), 10 mM Tris (pH = 7.4). Viral dsRNA bands were visualized on 0.8% agarose gel after DNase I enzyme (Fermentas, Life Sciences) treatment according to the manufacturer's instructions (Hajjaran et al., 2016).

RNA extraction and synthesis of cDNA

Total RNA from 1-5 $\times 10^6$ promastigotes in the stationary phase was isolated according to the manufacturer's instructions (Favorgen RNA extraction kit, Favorgen Biotech). Isolated RNA was resuspended in 60 μL of nuclease-free water. The quantity of the extracted RNAs was verified by a NanoDrop spectrometer (Thermo Scientific Fisher, USA). Based on the quantity of the extracted RNA, 1 to 2 μL were used for cDNA synthesis. The cDNA was then synthesized with a combination of oligo (dT) 18 and random hexamer primers using the cDNA Synthesis Kit (YTA, Iran with Cat No: YT4500) according to the manufacturer's instructions.

Semi-nested PCR

Semi-nested PCR assays for detection of LRV-2 were performed with set primers for the first stage LRV F1 (5' TGT AAC CCA CAT AAA CAG TGT GC 3') and LRV R (5'ATT TCA TCC AGC TTG ACT GGG 3') that amplified a 526-bp external partial sequence of the RdRp gene. To amplify the 315-bp internal fragment of the RdRp gene, specific primers LRV F2 (5' AGG ACA ATC CAA TAG GTC GTG T 3') and LRV R primers were used (Hajjaran et al., 2016). The PCR amplification was carried out in a 25 μL PCR reaction mixture consisting12.5 μL of master mix (Ampliqon, Denmark), 10 pmol each of forward and backward primers, and 1 μL of cDNA. The final volume was made 25 μl by the addition of nuclease-free water.

The PCR conditions for the semi-nested PCR in both stages consisted of initial denaturation of DNA strands at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 35 s, primer annealing at 60 °C for 35 s and strand elongation at 72 °C for 45 s. A final extension of the strands was made at 72 °C for 5 min. The PCR products were confirmed by visualization on 1.2% agarose gel stained with SYBR safe gel stain (Thermo Fisher Scientific, USA) for visualization of corresponding size PCR products. Standard reference strains of LRV 2 [LRV2/IR/2014/HM-2 (Acc. No. KP054245)] were used as positive controls.

Sequencing, phylogenetic analysis and haplotype network

PCR products of the RdRp gene related to 15 unique LRV2 isolates were purified and sequenced using an ABI PrismTM 3730 Genetic Analyzer (Applied Biosystems, Foster City, California, USA) by the Macrogen Company (Seoul, South Korea). Multiple alignments of viral sequences were performed using BioEdit, version 7.0.5 and examined using the program Mega 7 .0.7. To estimate phylogenetic trees for the LRV data sets we selected the maximum likelihood (ML) methods using Kimura 2-parameter models in MEGA 7 .0.7. In addition, bootstrap resampling analysis (1,000 replications) was used to assess branch confidence in clades in each tree (Kumar et al., 2016). Five LRV1 samples including *L. (V.) guyanensis* and *L. (V.) braziliensis* were also added to the phylogenetic analysis for comparison. Furthermore, *Trichomonas vaginalis* virus (TVV) was used as outgroup to construct the phylogenetic tree. The number of segregating sites, diversity indices (Haplotype diversity and Nucleotide diversity) and neutrality indices (Tajima's D and Fu's Fs tests) were estimated by DnaSP software version 5.10 (Rozas et al., 2003). A network of LRV2 haplotypes based on the sequences of RdRp was drawn by PopART software version 7.1 (Bandelt et al., 1999).

Results

Identification of Leishmania *isolates based on the nagt-PCR-RFLP*

Leishmania DNA was detected in all 85 isolates obtained from CL patients and amplification of *Leishmania*DNA exhibited a single 1450–60 bp fragment of the N-acetyl glucosamine-1-phosphate transferase (*nagt*) gene, which was consistent with the product size of *Leishmania* genus. Digestion with the ACC1 enzyme produced two bands of 500 and 950 bp for *L. major* and two bands of 680 and 780 bp for *L. tropica*, respectively. Based on the ACC1 enzyme digestion pattern , 83 out of 85 isolates obtained from CL patients were identified as *L. major* and 2 as *L. tropica*.

Detection of LRV2 in Leishmania *isolatesbased on the RdRp-semi-nested PCR*

Based on the amplification of the 526-bp and 315-bp products in the first and second rounds of RdRp-semi-nested PCR, 59 of 85 (69.4 %) clinical cultures were LRV2 positive and 26 were LRV2 negative. LRV2 was detected in 58/83 cases of *L. major* and 1/2 cases of *L. tropica* infections. It should be noted, the LRV2- *L. tropica* isolate was first reported in Iran. The presence of LRV2 was confirmed using a dsRNA extraction, which recognizes specifically a distinct band of about 5.3 kb indicative of viral dsRNA (Figure 2).

Phylogenetic characteristics and haplotype diversity of LRV2 isolates

Sequencing of the 15 unique samples carried out by Sanger sequencing method and sequences were deposited to GenBank

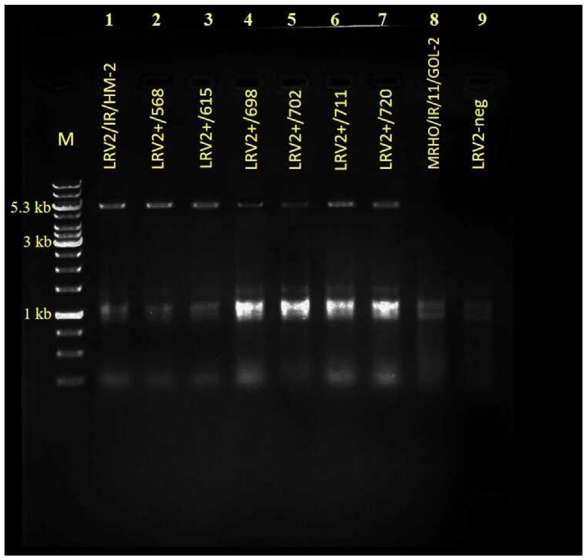

Figure 2. Visualization of viral dsRNA by gel electrophoresis. M: 1 kb ladder marker, Line 1: Positive control of LRV2 (LRV2/IR/2014/HM-2), Lines 2–7: Samples of LRV2 positive (current study), Line 8: Negative control of LRV 2 (MRHO/IR/11/GOL-2), Line 9: Sample of LRV2 negative (current study).

under the accession numbers (GenBank Acc. No: MT268281-MT268295). Sequences were 95-99 % similar to the partial sequence LRV2 isolated from Iran, Turkey, Ethiopia and complete genome LRV2 isolate from Uzbekistan and Turkmenistan (Figure 3). The intra-species difference was observed among the current RdRp sequences isolates (LRV2) and the overall mean distance between present isolates was calculated to be 0.022. Among LRV2 sequences in this study, high differences were observed between the isolate of LRV2/IR/GOL/711 with isolates of LRV2/IR/GOL/715 and LRV2/IR/GOL/720 (0.0636) (Table 1).

To determine haplotype diversity, LRV2 sequences from this study with other OWL ones from Iran (n = 3), Turkey (n = 10), Uzbekistan (n = 2) and Turkmenistan (n = 1) were analyzed. In current study, 10 haplotypes were reported but totally, 19 haplotypes of LRV2 were identified that 16 haplotypes in LRV2-*L.major*, 2 haplotypes in LRV2- *L.tropica* and 1 haplotype in LRV2- *L. infantum* (Table 2). The minimum spanning network was conducted in order to discern a genealogical relationship among the haplotypes (Figure 4). The MK246759.1 was the most common haplotype (n = 7) in the population network that belong to LRV2-*L. tropica* strains. The diversity and neutrality indices were calculated based on the RdRp gene sequences (Table 3). In the 19 haplotypes of LRV2, the relative high haplotype diversity belonged to LRV2-*L. major* (Hd: 0.957), while haplotype diversity for LRV2-*L. tropica* and LRV2-*L. infantum* were (Hd: 0.250 and Hd: 0), respectively. Nucleotide diversity was ranging from 0.02602 for LRV2- *L. major* to 0.00514 for LRV2-*L. tropica* and 0 for LRV2-*L. infantum*. Negative values were observed for both Fu's Fs test and Tajima's D test for sequences from LRV2-*L. major* and LRV2-*L. tropica* (Table 3).

Discussion

CL is an endemic parasitic disease in Iran, in which two types of CL including ACL and ZCL occur in 25 of the 31 Iranian provinces (Ghatee et al., 2020; Yaghoobi-Ershadi et al., 2004). Golestan Province is one of the oldest ZCL foci in Iran, which has a border with Turkmenistan (Sofizadeh et al., 2016). In recent years, ZCL cases have been reported increasingly from different areas of Golestan Province, northeast Iran. A retrospective study of

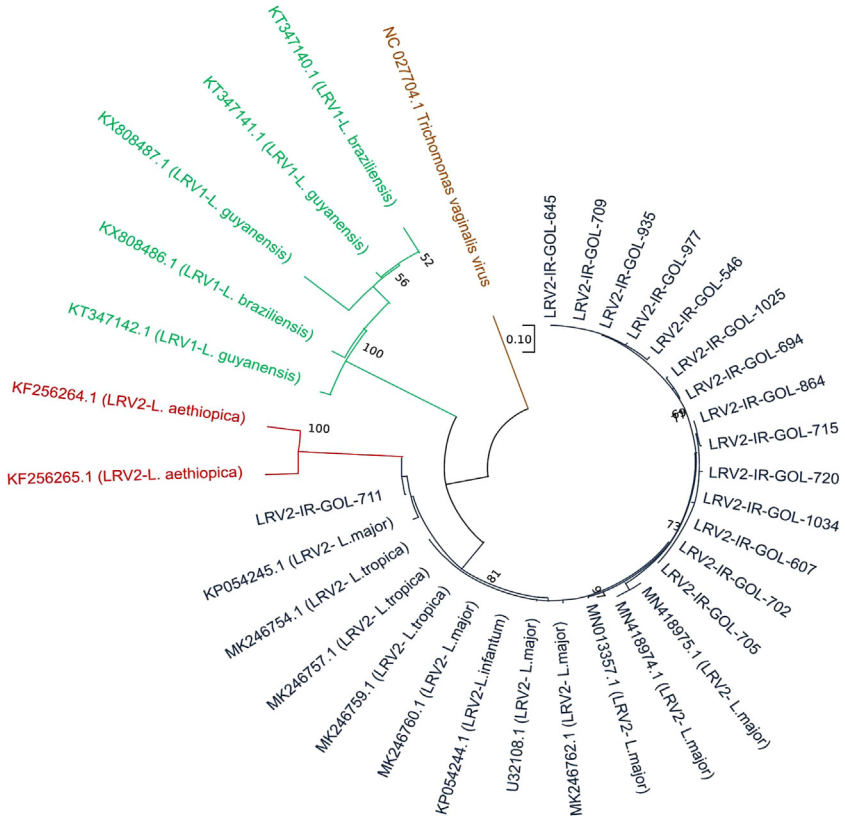

Figure 3. Phylogenetic tree of RdRp gene sequences of *Leishmania* RNA virus from CL patients in the present study and other LRVs from the NWLand OWL species obtained from GenBank.

Table 1
Diversity between the current LRV2 based on the RdRp gene.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	LRV2/IR/GOL/935															
2	LRV2/IR/GOL/864	0.0220														
3	LRV2/IR/GOL/1025	0.0223	0.0306													
4	LRV2/IR/GOL/1034	0.0182	0.0035	0.0264												
5	LRV2/IR/GOL/977	0.0035	0.0259	0.0261	0.0219											
6	LRV2/IR/GOL/546	0.0107	0.0257	0.0342	0.0297	0.0144										
7	LRV2/IR/GOL/645	0.0000	0.0220	0.0223	0.0182	0.0035	0.0107									
8	LRV2/IR/GOL/607	0.0182	0.0035	0.0264	0.0000	0.0219	0.0297	0.0182								
9	LRV2/IR/GOL/694	0.0107	0.0183	0.0109	0.0145	0.0143	0.0218	0.0107	0.0145							
10	LRV2/IR/GOL/702	0.0182	0.0035	0.0264	0.0000	0.0219	0.0297	0.0182	0.0000	0.0145						
11	LRV2/IR/GOL/705	0.0182	0.0035	0.0264	0.0000	0.0219	0.0297	0.0182	0.0000	0.0145	0.0000					
12	LRV2/IR/GOL/709	0.0000	0.0220	0.0223	0.0182	0.0035	0.0107	0.0000	0.0182	0.0107	0.0182	0.0182				
13	LRV2/IR/GOL/711	0.0499	0.0593	0.0600	0.0547	0.0541	0.0544	0.0499	0.0547	0.0458	0.0547	0.0547	0.0499			
14	LRV2/IR/GOL/715	0.0259	0.0035	0.0346	0.0071	0.0297	0.0295	0.0259	0.0071	0.0220	0.0071	0.0071	0.0259	0.0636		
15	LRV2/IR/GOL/720	0.0259	0.0108	0.0346	0.0071	0.0297	0.0378	0.0259	0.0071	0.0220	0.0071	0.0071	0.0259	0.0636	0.0145	

*Differences between isolate of LRV2/IR/GOL/711(13) with isolate LRV2/IR/GOL/715 (14) is 0.0636.

Table 2
Haplotype, isolates, country and host of *Leishmania* RNA virus 2, using the RNA dependent RNA polymerase (RdRp) sequences.

Haplotype	Haplotype. no	Isolates	Country	Host	Ref.
Hap_1	3	LRV2/IR/GOL/935	Iran	*L. major*	This study
		LRV2/IR/GOL/645	Iran	*L. major*	This study
		LRV2/IR/GOL/709	Iran	*L. major*	This study
Hap_2	1	LRV2/IR/GOL/864	Iran	*L. major*	This study
Hap_3	1	LRV2/IR/GOL/1025	Iran	*L. major*	This study
Hap_4	4	LRV2/IR/GOL/1034	Iran	*L. major*	This study
		LRV2/IR/GOL/607	Iran	*L. major*	This study
		LRV2/IR/GOL/702	Iran	*L. major*	This study
		LRV2/IR/GOL/705	Iran	*L. major*	This study
Hap_5	1	LRV2/IR/GOL/977	Iran	*L. tropica*	This study
Hap_6	1	LRV2/IR/GOL/546	Iran	*L. major*	This study
Hap_7	1	LRV2/IR/GOL/694	Iran	*L. major*	This study
Hap_8	1	LRV2/IR/GOL/711	Iran	*L. major*	This study
Hap_9	1	LRV2/IR/GOL/715	Iran	*L. major*	This study
Hap_10	1	LRV2/IR/GOL/720	Iran	*L. major*	This study
Hap_11	1	KP054244.1	Iran	*L. infantum*	Hajjaran et al. (2016)
Hap_12	1	MK246762.1	Turkey	*L. major*	Nalçacı et al. (2019)
Hap_13	1	MK246761.1	Turkey	*L. major*	Nalçacı et al. (2019)
Hap_14	1	MK246760.1	Turkey	*L. major*	Nalçacı et al. (2019)
Hap_15	1	KP054245.1	Iran	*L. major*	Hajjaran et al. (2016)
Hap_16	7	MK246759.1	Turkey	*L. tropica*	Nalçacı et al. (2019)
		MK246758.1	Turkey	*L. tropica*	Nalçacı et al. (2019)
		MK246757.1	Turkey	*L. tropica*	Nalçacı et al. (2019)
		MK246756.1	Turkey	*L. tropica*	Nalçacı et al. (2019)
		MK246755.1	Turkey	*L. tropica*	Nalçacı et al. (2019)
		MK246754.1	Turkey	*L. tropica*	Nalçacı et al. (2019)
		MK246753.1	Turkey	*L. tropica*	Nalçacı et al. (2019)
Hap_17	1	MN013357.1	Iran	*L. major*	Hajjaran et al. (2016)
Hap_18	2	MN418975.1	Uzbekistan	*L. major*	Kleschenko et al. (2019)
		MN418974.1	Uzbekistan	*L. major*	Kleschenko et al. (2019)
Hap_19	1	U32108.1	Turkmenistan	*L. major*	Scheffter et al. (1995)

routinely collected data from the Health System of Golestan Province (during 2010 to 2017 years) was indicated an increasing trend in the number of cases from the region (Jorjani et al., 2019). The first presence of LRV2 was reported from *L. major* in Turkmenistan in 1995 (Scheffter et al., 1995). In addition, previous evidence research the presence of LRV2 in Iran (Hajjaran et al., 2016). Hence, to the best of knowledge, this is the first haplotype and phylogenetic based study on the LRV2 strains from CL patients in a known ZCL focus in Iran.

We surveyed 85 samples of *L. major* and *L. tropica* isolated from infected patients, out of the 85 isolates, 59 samples were LRV2 positive. This study represents the first report of LRV2 in *L.tropica*in Iran and the second report of LRV2 in *L. major*, following the Hajjaran et al.'s study (Hajjaran et al., 2016). There is no comprehensive and epidemiological study in the OWL region regarding LRV2 in *Leishmania* spp., isolates. However, a study reported the presence of LRV2 in *L. aethiopica* human isolates in Ethiopia, in which 5 out of 11 *L. aethiopica* isolates were LRV2 positive (Zangger et al., 2014). In the study conducted by Hajjaran, out of 50 *Leishmania* isolated from CL and VL patients in different parts of Iran, only two isolates, *L. major* and *L. infantum* isolates were LRV2 positive (Hajjaran et al., 2016). Furthermore, investigate the presence of LRV2 in *Leishmania* species from Turkey indicated 7of the 24 *L. tropica* and 3 of the 3 *L. major* isolates were LRV2 positive (Nalçacı et al., 2019). In a recently published article, two isolates of LRV2 detected in 3 *L. major* isolates from human patients with CL in south Uzbekistan (Kleschenko et al., 2019). Considering the fact that the first LRV2 described in *L. major* species from Turkmenistan, and recent studies showed a high presence of the LRV2 was also detected among *L. major* species. Thus, it seems that

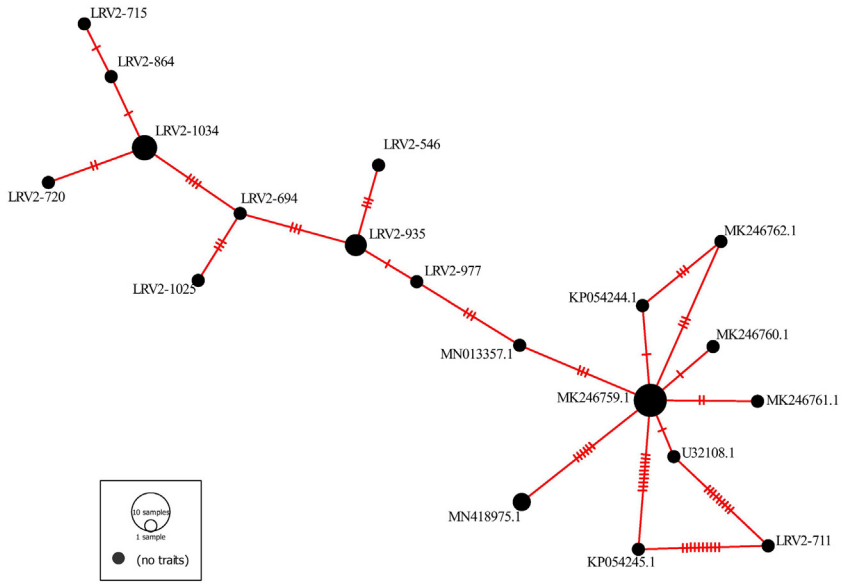

Figure 4. RNA-dependent RNA polymerase sequences (RdRp) haplotype networks in LRV2 from different geographical foci of Old World leishmaniasis.

Table 3
The diversity and neutrality indices of LRV2 populations from different species of Old World leishmaniasis based on RdRp gene.

Species	Diversity parameters						Neutrality indices	
	n	Hn	Parsimony informative sites	Singleton variable sites	(Hd)	(Nd)	Tajima's D	Fu's Fs statistic
L. major	22	16	21	20	0.957	0.02643	−1.22659	−4.278
L. tropica	8	2	0	6	0.250	0.00514	−1.63982	2.822
L. infantum	1	1	N.A.	N.A.	0.000	0.00000	N.A.	N.A.

* Hn: number of haplotypes; Hd: haplotype diversity; Nd: nucleotide diversity; N.A.: Not available.

the LRV2 is frequently detected from *L. major* species, which is consistent with the findings of our study.

In the meta-analysis study conducted by Saberi and colleagues, the global prevalence of LRVs was estimated to be 26.2%, which ranged from 10 % in Peru, to 70% in Brazil, and 74% in the French Guiana (Saberi et al., 2019). The results of these experiments indicated the 69.4 % (59/85) of *Leishmania* isolates were LRV2 positive. This difference might be attributed to the fact that the geographical origin of the *Leishmania* isolates. In this study, we report the high presence of LRV2 in Golestan Province, different factors could be regarded as the reasons, including tropical climate conditions, ecologic and epidemiologic aspects, and population movement to Turkmenistan for trade (Ghatee et al., 2019). These topic support the hypothesis that for the first time the LRV2 have entered Iran from Turkmenistan. In order to support this hypothesis, the partial sequence of the LRV2 isolates using BLASTn analysis revealed that 95-97% similarity to LRV2 from *L.major* in Turkmenistan (Acc. No: U32108.1). In addition, phylogenetic analysis, ecological changes and weather condition, common borders with Turkmenistan can support in this hypothesis. It should be noted that further studies such as whole genome sequencing of LRV2 and determination of its genetic map are needed to prove the hypothesis.

In the next step, the isolates were identified as LRV2 by phylogenetic analysis based on the RdRp sequence, an approach proven to be useful for the molecular characterization of LRV 2 (Hajjaran et al., 2016). Moreover, the phylogenetic analysis of current LRV2 sequences was similar to those of LRV2 reference isolates from OWL species (Iran, Turkey, Turkmenistan, Uzbekistan and Ethiopia) (Hajjaran et al., 2016; Nalçacı et al., 2019; Scheffter et al., 1995; Kleschenko et al., 2019; Zangger et al., 2014). From a phylogenetic perspective, the LRVs sequences in the phylogenetic tree can be grouped distinctly into two main clusters, one containing the LRV 1 sequences while the other LRV2 sequences, as we expected. Interestingly, the current sequence of RdRp gene showed several different nucleotides in distinct LRV2 isolates. This phenomenon revealed the genetic variation of the LRV2 genome (Scheffter et al., 1995). In the previous studies, the LRV1 type is divided into 14 subtypes (LRV1-1 to LRV1-14) (Tarr et al., 1988; Guilbride et al., 1992; Widmer et al., 1989). Interestingly, the LRV1-1 and LRV1-4 sequences share 77% overall nucleotide identity between themselves (Scheffter et al., 1995), representing genetic variation of the LRV, which is consistent with the findings of the current study (Table 1). Another study conducted in Uzbekistan reported the complete sequence of two *Leishmania* RNA Virus 2 found in L. major isolated from patients with CL. Results from this study showed that 170 nucleotide /40 amino acid and 174 nucleotide/41 amino acid differences between Uzb1/ASKH and Uzb2/ASKH sequences, respectively (Kleschenko et al., 2019). Moreover, this result was in consistent to that of Tirera et al, which indicated that 24 LRV1 full-length coding sequences presenting among themselves a significant genetic diversity (Tirera et al., 2017). Based on the findings of aboved-mentioned studies, it seems that the genetic diversity in the RdRp gene is relatively high.

The haplotype diversity value based on RdRp sequences revealed the degree of diversity within the LRV2 isolates. Relative high levels of haplotype and nucleotide diversity were observed in LRV2-*L. major* and this population expansion was evident from Fu's Fs test for LRV2-*L. major* sequences, where a negative Tajima's D test implies purifying selection. Indeed, a recent population expansion was evident from Fu's Fs test for LRV2-*L. major*

sequences, where a negative Tajima's D test implies purifying selection. On the other hand, positive Fs value observed in LRV2-*L. tropica* sequences are suggestive of a recent population bottleneck. The fact that current LRV2 populations shared 10 haplotypes, which probable is these haplotypes emerged in different regions and spread into Golestan Province. The high number of haplotypes reported in this study is probably due to genetic diversity among the isolates in this study. However, more extensive sampling and more in-depth molecular studies in other regions are needed to confirm this assumption.

A relative high value of haplotype diversity among LRV2-*L. major* may be involved in exacerbating of *L. major* pathogenicity including the creation of metastatic lesions, parasite replication, and developing treatment failure in clinical isolates. To this end, we have conducted a study for this topic and its results will be published in the future.

In recent study, conducted by Atayde et al. finding indicated that a LRV spreads by exploiting a mechanism used for cell-to-cell connection, a discovery that could introduce the way to new vaccines against severe form of the disease. In above-mentioned study, these researchers state that LRV1 hides in tiny vessels, known as exosomes pathway. In future, the use of *Leishmania* exosomes containing the LRV could proposed as new vaccine candidate against leishmaniasis (Atayde et al., 2019).

Conclusion

This study is the first report on the presence of LRV2 in Golestan Province and it is the second one in Iran. We have developed a sensitive method to evaluate the presence of LRV2 in *Leishmania* positive clinical samples. To conclude, the high prevalence of *Leishmania* strains infected with LRV2, displaying the capability of *Leishmania* spp. to establish an endosymbiotic relationship with the *Leishmania* RNA virus simultaneously. The present study aligns with previous reports in that haplotype diversity exists between populations of this LRV2 strains. *Leishmania* isolates obtained from this study were from the patients confirmed with CL in Golestan Province, thus, the finding of this study may not be generalizable to other populations or other geographic areas of the Iran and neighboring countries.

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Ethical statement

This study was approved by the ethical principles and the national norms and standards for conducting Medical Research in Iran for the molecular identification of *Leishmania* RNAvirus2 (LRV2) in *Leishmania* parasites isolated from CL patients (IR.MAZUMS.REC.1398.344).

Conflict of interest

The authors declare that there is no conflict of interests.

Author contributions

RS, MF and HH developed the study design. RS and YD collected the samples. RS performed the experiments and wrote the first draft of the paper. MM, AA, NT, HZ and AB had full access to the data and take responsibility for the accuracy of the data analysis. MF and HH assisted with data interpretation. All authors read, revised, and approved the final draft.

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